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The structure of the third intracellular loop of the muscarinic acetylcholine receptor M_2 subtype

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Abstract **We have examined whether the long third intracellular loop (i3) of the muscarinic acetylcholine receptor M_2 subtype has a rigid structure. Circular dichroism (CD) and nuclear magnetic resonance spectra of M_2i3 expressed in and purified from *Escherichia coli* indicated that M_2i3 consists mostly of random coil. In addition, the differential CD spectrum between the M_2 and $M_2\Delta$i3 receptors, the latter of which lacks most of i3 except N- and C-terminal ends, gave no indication of secondary structure. These results suggest that the central part of i3 of the M_2 receptor has a flexible structure.**

Keywords: Circular dichroism; G protein-coupled receptor; Secondary structure

1. Introduction

The muscarinic acetylcholine receptor belongs to the superfamily of G protein-coupled receptors (GPCRs) and plays pivotal roles in signal transduction. It consists of five genetically defined subtypes, M_1–M_5. One of the structural features of muscarinic receptors is that each subtype contains a long third intracellular loop (i3) of 160–240 residues.

Studies based largely on mutagenesis have shown that the N- and C-terminal portions of i3 play a key role in the specificity of the coupling of muscarinic receptors to G proteins [1]. On the other hand, most of the central part of i3 can be deleted without impairing the ability of the M_2 receptor to couple with G proteins [2], indicating that only the N- and C-terminal portions of i3 are required for the interaction. Consistently, most GPCRs have a short i3 and lack its long central part. On the other hand, dopamine D_{2-4}, adrenergic α_{2A-2C}, and 5-hydroxytryptamine$_{1A}$ receptors, like muscarinic receptors, possess a long i3 with more than 100 residues. The question is raised if these long i3 loops have a common physiological function and similar tertiary structure, although their sequences are not similar.

One of the common functions of the long i3 is presentation of the sites for agonist- and/or second messenger-dependent phosphorylation [3–5]. For example, the M_2 receptor undergoes agonist-dependent phosphorylation in the i3 by GPCR kinase 2 followed by desensitization of the receptor [6,7]. The long i3 is also involved in protein–protein interactions. Regulator of G protein signaling 2 and Gβγ are reported to bind directly to the i3 of M_1 and M_3 receptors, respectively, attenuating the receptor activity [8,9].

To the best of our knowledge, however, it has not been reported whether the long i3 of any GPCRs has a physiologically significant tertiary structure. In the present study, we employed circular dichroism (CD) and nuclear magnetic resonance (NMR) to elucidate the structure of M_2i3 and found it to be random coil nature.

2. Materials and methods

DNA techniques. The cDNA predicted to encode the whole M_2i3 (His208–Thr388) was amplified by polymerase chain reaction, where a sequence encoding 6× His tag (H) was added at the downstream, and *Eco*RI and *Not*I sites were introduced to the 5′- and 3′-ends, respectively. The resultant DNA was digested and ligated to pGEX-6P-3 (Amersham Biosciences, Little Chalfont, UK) to construct pGEXM_2i3H, which produces glutathione *S*-transferase (GST)-M_2i3H (Fig. 1A and B). DNA sequence was confirmed with the Applied Biosystems 3100-Avant sequencer (Foster City, CA).

Purification of GST–M_2i3H, M_2i3H, and GST. *Escherichia coli* BLR(DE3) transfected with pGEXM_2i3H were grown aerobically at 37 °C for 12 h in LB medium (2 L) containing 0.1 mg/mL ampicillin. After mixed with 0.2 mg/mL ampicillin and 0.2 mM isopropyl-1-thio-β-D-galactoside, the cells were further cultivated at 25 °C for 12 h. The cells were harvested and resuspended in phosphate buffered saline (PBS)(50 mL) containing 50 μg/mL lysozyme, 1 μM pepstatin A, 0.28 μM E-64, 0.2 mM benzamidine, 10 μM leupeptin, 0.2% (w/v) Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride, and 0.02% (w/v) protamine. The cells were disrupted by sonication (4 cycles of 15 s on and 45 s off at level 4) with a Tomy UD-200 ultrasonic disruptor (Tokyo), and subjected to centrifugation. Column chromatography was performed with the ÄKTA system (Amersham Biosciences) at room temperature. The supernatant was loaded onto Glutathione Sepharose 4B (5 mL) and washed with 50 mM Tris–Cl, pH 8.0, containing 150 mM NaCl (50 mL). The column was detached from the system and incubated at 5 °C for 4 h after applying PreScission Protease (80 units). After incubation, it was reattached and washed with the buffer (15 mL). The eluted M_2i3H was then loaded onto HiTrap Chelating Sepharose (1 mL), washed with PBS containing 10 mM imidazole (10 mL), and eluted with PBS containing 500 mM imidazole (5.5 mL). The imidazole was removed with Superdex 200 10/300 GL (24 mL) preequilibrated with PBS. The fractions containing purified M_2i3H were concentrated (6 mL → 200 μL) with a Centricon YM-10

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Abbreviations: CD, circular dichroism; GPCR, G protein-coupled receptor; GST, glutathione *S*-transferase; i3, third intracellular loop; NMR, nuclear magnetic resonance; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TM, transmembrane

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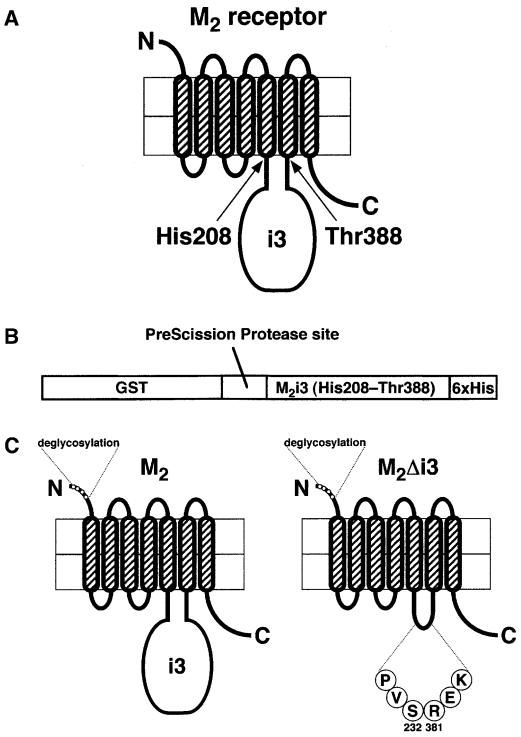

Fig. 1. Schematic diagrams of the M_2 receptors and the expression construct. (A) A putative topology of the M_2 receptor. (B) Construction of GST–M_2i3H. The sequence encoded by the multi-cloning site of pGEX-6P-3, Leu-Gly-Ser-Pro-Asn-Ser, was inserted between the PreScission Protease site (Leu-Glu-Val-Leu-Phe-Gln-Gly-Pro) and M_2i3H. Digestion with the protease leaves Gly-Pro. (C) The M_2 and M_2Δi3 receptors. Asn residues (2, 3, 6, and 9), which may undergo glycosylation, were replaced by Asp to avoid heterogeneity due to attachment of oligosaccharides. The central part of i3 (Pro233–Ser380), which contains all the phosphorylation sites, was deleted in M_2Δi3.

centrifugal filter unit (Millipore, Billerica, MA). Free GST was collected from Glutathione Sepharose 4B with 50 mM Tris–Cl, pH 8.0, containing 10 mM reduced glutathione after elution of M_2i3H. The cleavage step was omitted to prepare GST–M_2i3H. The N-terminal sequence of the purified M_2i3H was confirmed with Shimadzu PPSQ-21 (Kyoto) and Applied Biosystems 476A protein sequencers. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL) or by UV spectrometry using extinction coefficients calculated by the program ProtParam [10].

Expression and purification of the M_2 and M_2Δi3 receptors. Two recombinant baculoviruses were prepared, one with cDNA for the M_2 receptor and the other with cDNA for the M_2Δi3 receptor [11] (Fig. 1C). The receptors were expressed using the recombinant Sf9 cells as described [2]. Both receptors were purified by 3-(2′-aminobenzhydryloxy)tropane-based affinity chromatography, and the purified receptors were eluted as quinuclidinyl benzilate-bound form from a hydroxyapatite column, as described [2,12].

CD spectrum measurements. CD spectra were taken at 30 °C in a quartz cell with a 1-mm light path with a Jasco J-720 CD spectrophotometer (Tokyo). GST–M_2i3H, GST, and M_2i3H were adjusted to 0.1 mg/mL with PBS; M_2 and M_2Δi3 were adjusted to 0.1 mg/mL with PBS containing 0.2% (w/v) decylmaltoside and 0.05% (w/v) digitonin. CD intensities (mdeg) were recorded between 200 and 260 nm in steps of 0.05 nm, and converted to molar ellipticity θ values: deg cm^2 dmol^{-1} = mdeg × $10^{-3}_{(deg)}$/{0.1 mg/mL/(molecular weight)$_{(mol/L)}$ × 1/100$_{(dmol/cm^3)}$ × 0.1 cm}. The scan was repeated 2–3 times and averaged. To calculate the differential CD spectra between GST–M_2i3H and GST and between M_2 and M_2Δi3, the respective θ values for the latter proteins were subtracted from those for the former at all wavelength points. The α-helix content (%) was calculated using the equation, $100 \times ([\theta]_{222}/n) \times 1/\{-40000 \times (1 - 2.5/n)\}$, where n is the number of residues in the peptide [13].

NMR spectroscopy. Proton NMR spectra were acquired using Bruker Avance 800 MHz spectrometer equipped with cryoprobe (Bruker BioSpin, Karlsruhe, Germany). M_2i3H (82 μM) in 300 μl PBS containing 5% D_2O (v/v) was used for the measurements. One-dimensional experiments were performed with water suppression using WATERGATE pulse sequence with pulse field gradients [14]. Data were collected at 298 K, and were analyzed using XWINNMR 3.5 (Bruker).

3. Results

Purification of GST–M_2i3H, M_2i3H, and GST. We constructed GST–M_2i3H (Fig. 1B) to allow purification by both glutathione- and metal ion-based affinity chromatography. M_2i3H was isolated from GST–M_2i3H by cleavage of the PreScission Protease site which resides between GST and M_2i3H. These proteins were purified to homogeneity (Fig. 2). The band of M_2i3H (Fig. 2B, lane 2; calculated mass, 21 236) appeared above the band of GST (Fig. 2A, lane 3; calculated mass, 26 431). The N-terminal sequencing demonstrated that the isolated band shown in lane 2 of Fig. 2B possessed the stub sequence of the PreScission Protease site, followed by 6 amino acids encoded by the vector and the N-terminal sequence of M_2i3H (data not shown; see legend of Fig. 1B). Thus, the band was confirmed to be M_2i3H. There are many basic residues in N- and C-terminal portions of M_2i3H, and the isoelectric point (p*I*) of M_2i3H was estimated to be 9.34 from the primary structure [10]. On the other hand, the p*I* value of GST was estimated to be 5.73. The basic nature of M_2i3H may have caused slower migration compared with GST and marker proteins.

Secondary structures of GST–M_2i3H, GST, and M_2i3H. GST–M_2i3H and GST gave CD spectra typical of proteins containing secondary structures (Fig. 3A). Contrary to the CD spectra of the two proteins, the differential CD spectrum, which should represent the spectrum for the M_2i3H part of GST–M_2i3H, was close to that observed for random coil peptides. The helix contents of GST–M_2i3H, GST, and M_2i3H were calculated to be 15.7%, 23.4%, and 7.0%, respectively.

For isolated M_2i3H, the CD spectrum (Fig. 3B) was similar to the differential one (Fig. 3A), supporting the idea that M_2i3 has little secondary structure. The helix content of the purified M_2i3H was calculated to be 13.3%. The possibility that a small

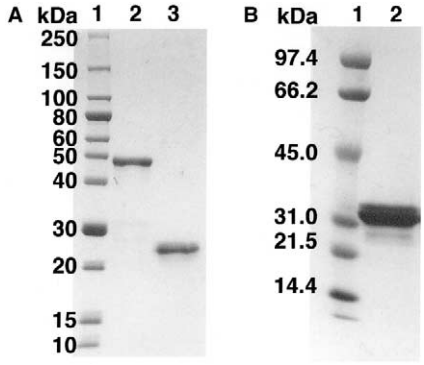

Fig. 2. Purification of the proteins. The purified GST–M_2i3H (A, lane 2), GST (A, lane 3), and M_2i3H (B, lane 2): 0.5 μg (A) and 2.6 μg (B) of proteins were subjected to SDS–PAGE and stained with Coomassie brilliant blue R-250. Calculated mass of GST–M_2i3H is 47 649.

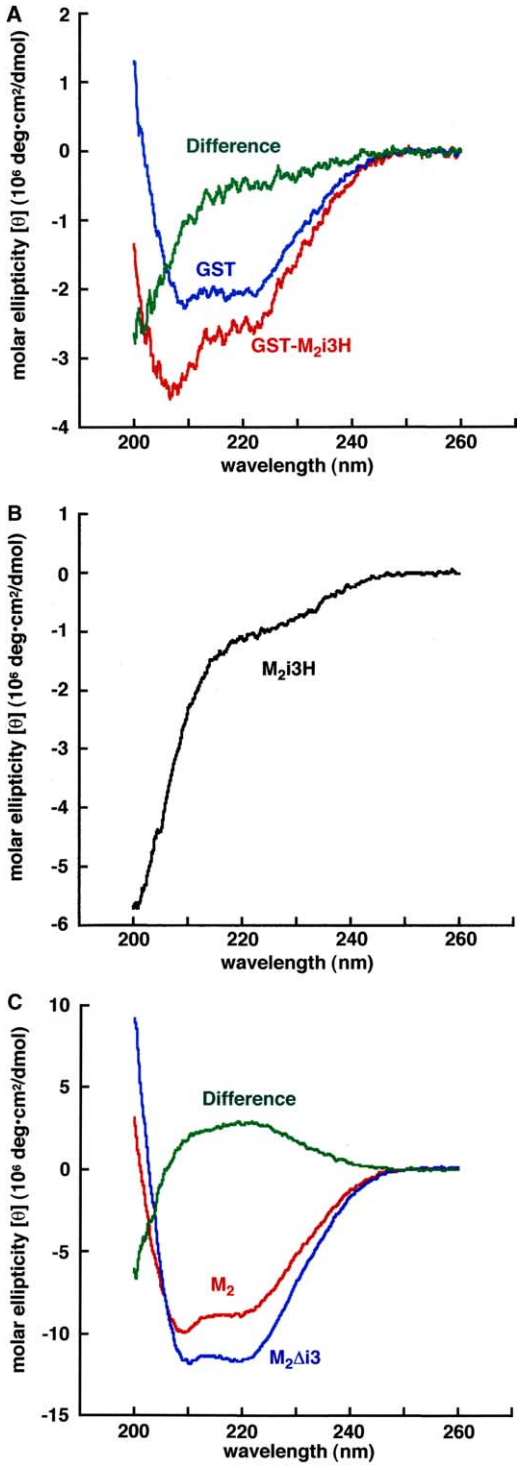

Fig. 3. CD spectra of the proteins. (A) GST–M_2i3H, GST, and the difference. (B) The isolated M_2i3H. (C) M_2, $M_2\Delta$i3, and the difference.

amount of α-helix may exist in i3 is also reflected by the depressed pattern of the spectrum around 208–222 nm.

The random coil nature of M_2i3H was further confirmed by 1D NMR (Fig. 4). The resonances attributed to methyl groups of amino acid residues seem not to be found in the range 0.0–0.5 but rather in the range over 0.7 ppm (Fig. 4C). Many signals were found to accumulate around 8.0 ppm (Fig. 4B), which corresponds to the region of amide proton. These results suggest that the purified M_2i3H is not likely to have an ordered structure.

CD analysis of M_2 and $M_2\Delta i3$. The structure of the isolated peptide produced in *E. coli* may not necessarily represent the structure of the same sequence in the intact receptor: the constraints imposed by covalent attachment to the transmembrane (TM) regions as well as the interaction with other cytoplasmic region(s) might result in stabilization of a certain conformational state of i3. To examine this possibility, we prepared an M_2 receptor mutant lacking most of i3 ($M_2\Delta$i3), which is known to show essentially the same pharmacological properties as the wild-type M_2 receptors [2,11] (Fig. 1C). Both M_2 and $M_2\Delta$i3 receptors were purified to homogeneity as assessed by gel chromatography (Superdex 200 10/300 GL) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; data not shown), as described previously [2,11,12].

Both M_2 and $M_2\Delta$i3 receptors exhibited the spectra typical of proteins containing secondary structures (Fig. 3C): the helix contents of M_2 and $M_2\Delta$i3 were calculated to be 46.6% and 90.7%, respectively. This is consistent with the widely accepted notion that GPCRs are composed of 7 TM α-helices. In fact, analysis of the crystal structure of bovine rhodopsin (PDB ID, 1F88) provided the following proportions of secondary structures (%): β-strand, 3.6; α-helix, 58.9; 3_{10} helix, 2.7; others, 34.9 [15]. The differential CD spectrum corresponding to i3 in the receptor, however, showed no signs of secondary structure. The estimation of the helix content in i3 (Pro233–Ser380) gave a negative value, suggesting that no α-helix was present in the central part of i3.

4. Discussion

In the present study, we have shown that M_2i3 has little secondary structure: the helix content in M_2i3H (His208–Thr388) purified from *E. coli* and in the central part of i3 (Pro233–Ser380) in the receptor was estimated to be 13.3% and 0%, respectively, from the mean residue ellipticity values at 222 nm ($[\theta]_{222}/n$).

In other studies, the N- and C-terminal portions of i3 in muscarinic receptors and other GPCRs have been shown to form α-helices which are extensions of TM5 and TM6, respectively, based largely on findings that a thorough mutagenesis of relevant residues showed the periodicity in tolerance to the substitutions or the lack of tolerance by disrupting putative α-helix by substitution with proline [1,16]. We subjected the M_2i3 sequence to prediction of the secondary structure using the two programs, Porter [17] and PSIpred [18], and found that the central part of i3 was predicted to be mainly coil by either program, α-helix and/or β-strand to lie near or adjacent to the N- and C-terminal regions, and the helix content to be 13.3% and 8.8%, respectively, by the two programs. These predictions are consistent with our present finding that there is a small amount of secondary structure in the full-length of isolated M_2i3 but no secondary structure in the central part of i3 in the M_2 receptor.

The structure determined for the isolated i3 may not represent the actual structure in the receptor in vivo, as i3 may be influenced by other intracellular loop(s) and/or the C-terminal tail. This has been shown not to be the case, however, for the intracellular loops of rhodopsin as follows. NMR analyses of the synthetic i1, i2, and i3 peptides of rhodopsin have revealed that

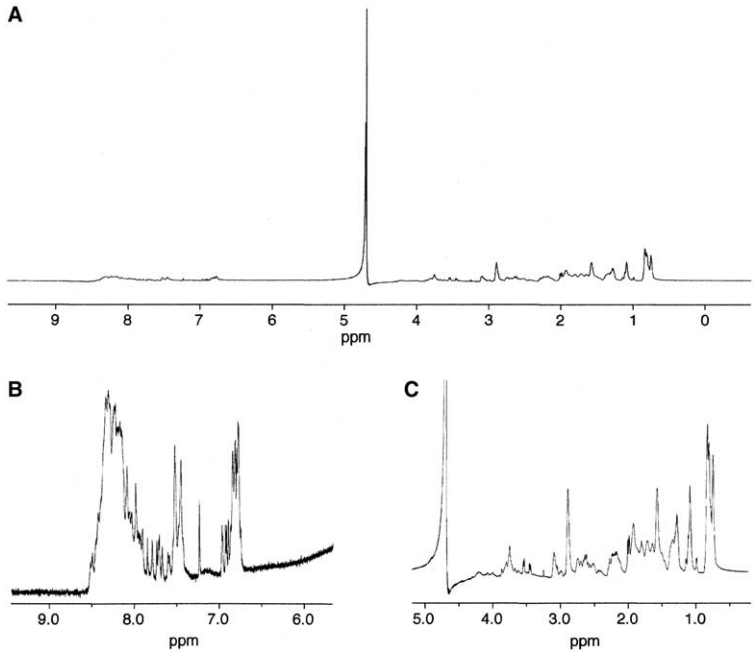

Fig. 4. ^{1}H 1D NMR of the isolated M_2i3H. The complete (A), expansion of the amide proton (B), and high field (C) regions.

these loops have an ordered structure containing an α-helix and/or β-turn and that both ends of each peptide are close together, thereby positioning the termini appropriately for connection of adjacent TM helices, as would occur in the intact receptor, even though TM helices were absent in these peptides [19,20]. The X-ray crystallographic analysis of rhodopsin, in which most of the residues of the loops were identified, verified that these cytoplasmic loops have an ordered structure rather than a random one [15]. In the present report also, essentially the same results were obtained for isolated i3 and the i3 in M_2 receptor, confirming that the central part of M_2i3 does not have secondary structure, and that other intracellular loops and/or the C-terminal end probably have little, if any, influence on its structure. The possibility remains, however, that i3 may form a definite structure when it binds cytosolic protein(s). It should be noted in this context that some proteins, which can constitute supramolecular complexes, form a cooperatively folded structure only after they meet the binding partner, though the isolated domains are intrinsically disordered [21].

In conclusion, the present results suggest that the central part of the third intracellular loop of the M_2 receptor (M_2i3) does not have secondary structure, and most probably has a flexible structure, though its N- and/or C-terminal regions may form α-helices. Several questions remain to be answered in the future: (1) Could this conclusion be generalized to the long i3 of other GPCRs? (2) Is the flexibility required for the function of the long i3? (3) Do C-terminal tails of many GPCRs, which have functions similar to those of the long i3, have a flexible structure?

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